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Phototoxicity and Differential Hepatotoxicity as Biological Indicators of Nitrosamine Activity

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A series of 23 nitrosamines were studied from the standpoint of evaluating their phototoxic and hepatotoxic properties as biological parameters for purposes of bioassay. With respect to differentiation between carcinogenic and noncarcinogenic nitrosamines, phototoxicity appears to be a nonspecific effect related to the photodecomposition of the nitroso group and dependent on the physical properties of the nitrosamine. The most phototoxic nitrosamine studied was N-nitrosocarbazole, but it possessed only approximately 1/100 of the toxicity of 7,10-dimethylbenz(c)acridine and 1/200 of the toxicity or rose bengal which were used as reference standards.

A more promising approach to bioassay of nitrosamines lies in their ability to induce or inhibit liver microsomal enzymes with resulting decreases or increases in pentobarbital sleeping times (PST) in mice. Those nitrosamines known to be carcinogenic which were investigated prolonged PST, while all of the known noncarcinogenic nitrosamines studied shortened PST and some produced an increase in smooth endoplasmic reticulum in mouse liver hepatocytes. Dibutylnitrosamine, N-nitrosocarbazole and N-phenyl-N-nitrosobenzylamine had a dual effect on PST based on dosage. The combined effects of nitrosamines on liver enzyme induction as reflected in changes in PST and the anabolic and catabolic hepatotoxicity may be used to differentiate carcinogenic nitrosamines from noncarcinogenic nitrosamines in periods of time as short as 4 days whereas conventional bioassays require 30-270 days.

INTRODUCTION

The demonstration of the carcinogenicity of nitrosamines (Magee and Schoental, 1964; Magee and Barnes, 1967; Magee, 1971; Druckrey et al., 1967) has led to intensive biological and chemical research in this field because of widespread environmental implications. Nitrosamines have been reported as contaminants in foods and feeds treated with sodium nitrite (Thewlis, 1968; Ender and Ceh, 1968; Hedler and Marquardt, 1968; Freimuth and Glaser, 1970; Fazio et al., 1971) and in cigarette smoke (Serfontein and Hurter, 1966; Serfontein and Smit, 1967; Neurath, 1967; Pailer and Klus, 1971). In addition, the ability of certain plants and vegetables to sequester nitrates and nitrites from the soil provides a massive reservoir of nitrosamine precursors in the food and feed chain and poses a presumptive hazard to man.

Despite recent advances in the estimation of trace amounts of nitrosamines in foods (Walters, 1971) involving thin layer chromatography, gas chromatography, spectrophotometry, mass spectrometry, polarography, and ultraviolet light-induced nitrite release, the presence of interfering artefacts precludes any general chemical method for the separation and identification of volatile and nonvolatile nitrosamines in a given food matrix. Attempts at bioassay involving the Pekin duckling (Carlton et al., 1966) require a 30 day feeding trial and considerable quantities of contaminated feed.

In this study the phototoxic properties of nitrosamines in protozoa and the anabolic and catabolic hepatotoxicity of nitrosamines in the mouse liver are considered from the standpoint of differentiation between carcinogenic and noncarcinogenic nitrosamines in the development of more sensitive bioassay procedures.

A variation of the technique originally described by Epstein et al., (1963) was used for phototoxicity tests. Tetrahymena pyriformis T was used as the test organism and this protozoan was grown axenically in 1% Bacto-peptone, 0.05% Difco[2] yeast extract, and 0.25% glucose for 2 days. The light source consisted of a 275 watt RS Sunlamp positioned 8.5 inches above the sample and the heat produced by lamp was filtered out by water in a 2 liter flat bottomed Pyrex crystallizing dish placed 3.5 inches above the sample. The spectral distribution and power output of the lamp are described elsewhere (Nishie et al., 1968). Prior to light exposure, 0.025 ml of the Tetrahymena culture and 0.025 ml of a freshly prepared aqueous nitrosamine solution were delivered by microsyringe to the 3 wells of a glass concavity slide. Three sample slides were exposed to light for a period as long as 20 min simultaneously and a fourth slide was kept in the dark to serve as a dark control.

Solid nitrosamines were first dissolved in a small volume of dimethylsulfoxide and then diluted with water. Three or 4 concentrations of nitrosamine were used to establish the dose required to kill 100% of the Tetrahymena in 10 min ($LD_{100}^{10'}$) exposed to the light source. Nitrosamine doses were plotted on the logarithmic scale and times for 100% kill of the Tetrahymena on the linear scale of semi-log paper in deriving the dose-response curve used to estimate the $LD_{100}^{10'}$. The nitrosamines used in this study were either synthesized by one of the authors or purchased commercially. Rose bengal and 7,10-dimethylbenz(c)acridine were used as reference phototoxic compounds. The photodynamic activity expressed as $LD_{100}^{10'}$ in the tables is the average of 6 determinations.

Swiss Webster male mice weighing 18-20 grams were used to determine the hepatotoxicity of the nitrosamines and their indirect biochemical effects on the liver as monitored by the measurement of pentobarbital sleeping times. Groups of 10 mice were used at each dose level of nitrosamine until a minimal dose was found which significantly altered pentobarbital sleeping time. The nitrosamines were dissolved in olive oil and administered orally (po) by gavage for 4 consecutive days at a dose of 5 ml/kg/day. One control group of 10 mice of each new shipment received an equivalent amount of olive oil po for 4 days and one control group only Purina Mouse Chow and water for 4 days prior to pentobarbital administration. As positive controls of liver microsomal enzyme induction, groups of 10 mice were given phenobarbital sodium po dissolved in olive oil for 4 days prior to pentobarbital treatment.

On the fifth day, all mice were given an aqueous solution of pentobarbital (100 mg/kg) intraperitoneally (ip). The mean sleeping time and its standard error were determined for all groups and the Student "t" test was used for establishing the significance of differences between means of nitrosamine treated and control animals receiving only olive oil. At the conclusion of the experiment, the mice were sacrificed and livers removed for histological and electron microscopic examination.

Photographs of liver sections stained with haemotoxylin-eosin were taken with a Zeiss phase contrast microscope with Polaroid attachment. In the preparation of samples for electron microscopy, tissue slices 1-2 mm thick were taken from the left medial lobe and fixed for 1 hr in 1% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.3, at 4°C. The slices were then cut into 1 mm^3 blocks, fixed 1 hr in fresh buffered glutaraldehyde, washed 15 min in 0.1 M cacodylate buffer, pH 7.3, containing 5% sucrose, and post-fixed for 1 hr in 1%

osmium tetroxide in 0.1 M cacodylate buffer, pH 7.3, at 4°C. The tissues were dehydrated in graded alcohols and embedded in Maraglas (Freeman and Spurlock, 1962). Sections were cut using a Porter-Blum MT-2 ultramicrotome equipped with a glass knife, mounted on Athene copper grids, and stained with 2% uranyl acetate and lead citrate (Reynolds, 1963). Sections were examined using a Hitachi HU-12 electron microscope operating at 75KV.

The immediate effects of nitrosamines on pentobarbital sleeping times were studied by giving a single dose of nitrosamine dissolved in olive oil po 2 hrs prior to administration of pentobarbital sodium at a level of 100 mg/kg ip. Controls for this study were given 5 ml/kg of olive oil po 2 hrs before pentobarbital administration.

RESULTS

(A) Photodynamic Effect of Nitrosamines on Tetrahymena

The nitrosamines used in this study are listed in Table 1, with their UV absorption maxima which were obtained either from the literature (Druckrey et al., 1967) or determined by one of the authors.

In Table 2, the photodynamic lethality to Tetrahymena, expressed as $LD_{100}^{10'}$ of the nitrosamines is listed with molecular weights and water solubilities. There was no difference in phototoxicity between diethylnitrosamine (no. 2) and the cyclic N-nitrosopyrrolidine (no. 20) both of which contain 4 carbon atoms. Of the nitrosamines containing 5 carbon atoms, N-ethyl-N-nitrosopropylamine (no. 14), N-methyl-N-nitrosobutylamine (no. 11), and N-nitrosopiperidine (no. 21) had potency values which differed significantly (9.5, 11.1 and 5.8 mM/L, respectively). Diallylnitrosamine (no. 3) with 6 carbon atoms was roughly 3 times as phototoxic as the dipropyl (no. 4) and ethylbutyl (no. 15) analogs. Condensation of the benzene rings in N-nitrosocarbazole (no. 23) had the effect of doubling the phototoxicity when compared with diphenylnitrosamine (no. 6). The 3 noncarcinogenic nitrosamines, diphenyl (no. 6), dibenzyl (no. 7), and diallylnitrosamines (no. 3) were the most phototoxic of the symmetrical nitrosamines. The most phototoxic compound in the series was N-nitrosocarbazole (no. 23), but it appears very weak when compared with the reference phototoxicity compounds 7,10-dimethylbenz(c)-acridine (no. 25) and rose bengal (no. 24) which were 87 and 190 times more phototoxic, respectively. Lethal concentrations of nitrosamines exposed to light ($LD_{100}^{10'}$) had no effect on Tetrahymena when kept in the dark for the same duration.

(B) Effect of Nitrosamines on Pentobarbital Sleeping Time (PST) in Mice

Table 3 lists the nitrosamines in decreasing order of potency as measured by their ability to increase or decrease PST in mice after 4 days of oral administration. Many (8 of 16) of the nitrosamines caused loss of body weight at the minimal doses required to prolong PST significantly, whereas among those compounds which shortened PST only one (N-ethyl-N-nitrosobenzylamine) of 11 caused loss of body weight. In general, the alkylnitrosamines of low molecular weight and high water solubility were more effective in prolonging PST than aryl and cyclic nitrosamines with the exception of dibutylnitrosamine (no. 5). N-nitrosocarbazole (no. 23), N-phenyl-N-nitrosobenzylamine (no. 19) and dibutylnitrosamine (no. 18) shortened PST at low dosage levels but prolonged PST at near lethal doses. Three known noncarcinogenic nitrosamines [diallyl (no. 3), diphenyl (no. 6), and dibenzyl-nitrosamine (no. 7)] significantly shortened PST (Table 3 and 4). Diallyl-nitrosamine was the most active nitrosamine in shortening PST, but it was only 1/13 as effective as phenobarbital. A number of nitrosamines of unknown carcinogenic potential (N-nitrosoethylamine (no. 8), N-ethyl-N-nitrosoaniline (no. 13), N-butyl-N-nitrosoaniline (no. 18), N-phenyl-N-nitrosobenzylamine (no. 19), and N-nitrosocarbazole (no. 23)] also lowered PST. Among symmetrical and assymmetrical alkylnitrosamines, PST prolongation appeared to be inversely proportional to molecular weight whereas PST shortening had no apparent relationship to molecular size. Table 4 shows the effects of incremental increases in daily doses of diallylnitrosamine on PST. There was a dose related shortening of PST until the saturation point was reached.

When a single dose of diallylnitrosamine (100 mg/kg po) was given 2 hrs before pentobarbital, there was significant increase in PST. Treatment with a single dose of diallylnitrosamine (100 mg/kg po) 24 hrs before pentobarbital shortened PST to the same extent as the group which received a similar dose for 4 days. Single doses of dibenzyl- and diphenylnitrosamines also shortened PST with 24 hr pretreatment. Mice which did not receive any treatment before pentobarbital and the group which received only 5 ml/kg of olive oil showed no significant difference in PST. In Table 5, the effects of 2 hr pretreatment with a single dose of nitrosamine are shown. Of the 23 nitrosamines tested, 19 showed significant prolongation of PST at the 5% probability level and 4 were without effect.

(C) Histological Changes Caused by Nitrosamines in Mouse Liver

Representative micrographs of the centrolobular areas of livers of mice treated for 4 consecutive days with nitrosamines po are shown in Fig. 1. It can be seen that the liver of the mouse treated with 500 mg/kg/day of diphenylnitrosamine (B) did not differ from control (A) in the centrolobular area. A dose of 100 mg/kg/day of N-nitroso-N-ethylaniline (C) increased the density of hepatocytes. The remaining nitrosamines which shortened PST did not produce observable differences between control livers and livers of mice treated with 60 mg/kg/day of phenobarbital po. A dose of 30 mg/kg/day of diethylnitrosamine produced swollen hepatocytes in the centrolobular area (D) characteristic of toxic doses of carcinogenic nitrosamines. The same dose of N-methyl-N-propylamine (E) and N-ethyl-N-nitrosopropylamine (F) also caused swollen hepatocytes, but to a lesser degree. A dose of N-ethyl-N-nitrosobutylamine of 60 mg/kg/day also caused swelling limited to only a few hepatocytes in the centrolobular area. All known carcinogens tested in this study produced swelling of hepatocytes in centrolobular areas and necrosis at higher doses.

(D) Electron Microscopy

Hepatocytes from mice receiving olive oil appeared normal with respect to organelle sizes and shapes (Fig. 2A). Pretreatment with N-ethyl-N-nitrosopropylamine did not appear to alter quantity or shape of either rough or smooth endoplasmic reticulum (Fig. 2B). However, electron transparent bleb-like formations associated with mitochondria were noted (arrow).

Pretreatment of mice with diphenylnitrosamine (Fig. 3A) or N-ethyl-N-nitrosoaniline (Fig. 3B) resulted in greatly increased quantities of smooth endoplasmic reticulum distributed among granules of glycogen. Mitochondrial blebs were also noted, as were hypertrophy and pleomorphism of mitochondria. While rough endoplasmic reticulum (RER) of diphenylnitrosamine-pretreated mice appeared normal, the RER of mice receiving N-ethyl-N-nitrosoaniline was swollen.

DISCUSSION

All of the nitrosamines examined in this study proved to be phototoxic to Tetrahymena including those reported to be noncarcinogenic (Druckrey et al., 1967; Argus and Hoch-Ligeti, 1961). The correlation of carcinogenicity with phototoxicity reported for polycyclic hydrocarbons (Epstein et al., 1964) and not observed with nitrosamines may be due to the fact that the latter are not local carcinogens. The failure of phototoxicity per se to serve as a criterion for distinguishing carcinogenic nitrosamines from non-carcinogenic nitrosamines, severely limits this parameter as the basis for a potential bioassay method. In Table 5, a prolongation of PST is shown for 21 of 23 nitrosamines when single doses were administered 2 hrs before pento barbital. However, when these nitrosamines were administered for 4 consecutive days prior to injection of pentobarbital, either increased or decreased PSTs were observed (Table 3) suggesting inhibition or induction of pentobarbital metabolizing enzymes. All 3 of the noncarcinogenic nitrosamines (diallyl-, diphenyl-, and dibenzylnitrosamines) prolonged PST when a single dose was administered 2 hrs before pentobarbital, but shortened PST when administered 24 hrs before pentobarbital or for 4 daily consecutive doses preceding the barbiturate (Tables 3 and 4). This suggests that a 24 hr period is required for the induction of pentobarbital metabolizing enzymes. Further support for the concept of induction is provided by a limited number of electron micrographs in which an increase in the smooth endoplasmic reticulum (SER) of hepatocytes by diphenylnitrosamine or N-ethyl-N-nitroso-aniline is demonstrated (Figs. 3A and 3B).

The reported lack of correlation between local carcinogenic action and microsomal enzyme inducing ability of some 50 polycyclic hydrocarbons

(Arcos et al., 1961) has been questioned (Gelboin, 1967) because of the use of small single doses of test compounds. Both enzyme induction and proliferation of SER have been reported for 7,12-dimethylbenzanthracene and aflatoxin (Gelboin, 1967; Svoboda et al., 1966). The carcinogenic nitrosamines used in this study did not increase the amount of SER in short term experiments. In long term experiments, however, hypertrophied elements of SER were demonstrable after 289 days of low doses of dimethylnitrosamine administered in the drinking water of rats (Geil et al., 1968). The prolongation of PST (Table 3) by 11 known carcinogenic and 3 heretofore untested nitrosamines administered 4 days prior to pentobarbital in mice supports a similar finding (Stevenson and Greenwood, 1968) that high dose levels (30 mg/kg) of diethylnitrosamine inhibited hexobarbital metabolism of rats which resulted in prolonged hexobarbital sleeping time. Lower doses of dimethylnitrosamine (2 mg/kg) administered over a 114 day period (Ruddon, 1967) had no effect on hexobarbitone oxidase.

A classification of hepatotoxins (Madhaven et al., 1970) into anabolic or catabolic categories based on their ability to cause liver enlargement, increase in liver protein, and marked microsomal enzyme induction in the first case or inhibition of protein synthesis in the latter case may serve in characterizing the nitrosamines in this study. On the basis of this criterion, the catabolic nitrosamines would comprise those lengthening PST and having carcinogenic potential whereas those which shortened PST would be anabolic and noncarcinogenic. Three of the nitrosamines in the study about which there is no information concerning carcinogenicity (N-methyl-N-nitrosopropylamine, N-ethyl-N-nitrosopropylamine and N-ethyl-N-nitrosobutylamine) are possible carcinogens because they prolonged PST and produced

swelling of centrolobular hepatocytes characteristic of carcinogenic nitrosamines. In addition, N-ethyl-N-nitrosopropylamine caused blister-like budding in the mitochondria of hepatocytes (Fig. 2B) similar to that induced by aflatoxin (Svoboda et al., 1966) and dimethylnitrosamine (Mukherjee et al., 1963). Two of the known noncarcinogenic nitrosamines (diphenyl- and dibenzylnitrosamines) and the structurally similar compound of unknown carcinogenicity, N-nitrosocarbazole shortened PST suggesting that the latter is noncarcinogenic Recently Epstein and his coworkers (1971) reported that among polycyclic hydrocarbons there was a close association between photodynamic and enzyme-inducing activities. In the case of the nitrosamines there seems to be no correlation between photodynamic and enzyme-inducing activities because all 23 nitrosamines had photodynamic activity but only 9 of 23 were capable of enzyme induction. In conclusion, it would appear that differential hepatotoxicity (anabolic or catabolic) and the effects of nitrosamines on PST have potential in the bioassay of chemically unknown nitrosamines whereas the nonspecific photodynamic activity due mainly to the photodecomposition of the N-N=O group of the nitrosamines (Burgess and Lavanish, 1964; Chow, 1964) is of little value in this regard.

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FOOTNOTES

[1]Eastern Marketing and Nutrition Research Division, ARS, USDA, Wyndmoor, Pennsylvania 19118.

[2]Reference to a company or product name does not imply approval or recommendation of the product by the U. S. Department of Agriculture to the exclusion of others that may be suitable.

FIGURE LEGENDS

Fig. 1 Centrolobular areas of livers of mice treated for 4 consecutive days po with nitrosamines dissolved in olive oil and sacrificed on the fifth day. (Magnification 400x.)

A. 5 ml/kg/day olive oil (control liver)

B. 500 mg/kg/day diphenylnitrosamine

C. 100 mg/kg/day N-nitroso-N-ethylaniline - abundance of hepatocytes

D. 30 mg/kg/day diethylnitrosamine - swollen hepatocytes in the centrolobular area

E. 30 mg/kg/day N-methyl-N-nitrosopropylamine - swollen hepatocytes

F. 30 mg/kg/day N-ethyl-N-nitrosopropylamine - few swollen hepatocytes

Fig. 2 Electron micrographs of liver obtained from mice pretreated for 4 days po with nitrosamines dissolved in olive oil.

N = nucleus, M = mitochondrion, SER = smooth endoplasmic reticulum, RER = rough endoplasmic reticulum, S = sinusoid.

Uranyl acetate and lead citrate stain.

X 12,600

A. 5 ml/kg/day olive oil

B. 30 mg/kg/day N-ethyl-N-nitrosopropylamine. Arrow indicates mitochondrial bleb-like formation.

Fig. 3 Electron micrographs of liver obtained from mice pretreated for 4 days po with nitrosamines dissolved in olive oil.

N = nucleus, M = mitochondrion, SER = smooth endoplasmic reticulum, RER = rough endoplasmic reticulum.

Uranyl acetate and lead citrate stain.

x 12,600

A. 400 mg/kg/day diphenylnitrosamine

B. 100 mg/kg/day N-ethyl-N-nitrosoaniline

Nitrosamines

No.	Compound	U.V. Absorption Maxima (nm)
	Symmetrical Nitrosamines	
1	N-nitrosodimethylamine	332
2	N-nitrosodiethylamine	340
3	N-nitrosodiallylamine[a]	344
4	N-nitrosodipropylamine	345
5	N-nitrosodibutylamine	347
6	N-nitrosodiphenylamine[a]	360
7	N-nitrosodibenzylamine[a]	353
	Assymmetrical Nitrosamines	
8	N-nitrosoethylamine[b]	---
9	N-methyl-N-nitrosoethylamine	335
10	N-methyl-N-nitrosopropylamine[b]	361,374
11	N-methyl-N-nitrosobutylamine	361,378
12	N-methyl-N-nitrosoaniline	271
13	N-methyl-N-nitrosobenzylamine	374
14	N-ethyl-N-nitrosopropylamine[b]	365,376
15	N-ethyl-N-nitrosobutylamine	337
16	N-ethyl-N-nitrosoaniline[b]	386,404
17	N-ethyl-N-nitrosobenzylamine	365,377
18	N-butyl-N-nitrosoaniline	388,403
19	N-phenyl-N-nitrosobenzylamine[b]	---
	Cyclic Nitrosamines	
20	N-nitrosopyrrolidine	333
21	N-nitrosopiperidine	337
22	N,N'-dinitrosopiperazine	337
23	N-nitrosocarbazole[b]	---
	Reference Compounds	
24	Rose bengal	550
25	7,10-dimethylbenz(c)acridine	---

TABLE 2

Photodynamic Activity of Nitrosamines

No.[a]	R1 \ N-N=O / R2 R1	R2	Mol. Wt.	H_2O soluble g%	$LD_{100}^{10'}$[b] mM/L ± S.E.
24	Rose bengal		973	Very sol.	0.00011 ± 0.000027
25	7,10-DMB(c)A[c]		257	---	0.00024 ± 0.000015
23	N-nitrosocarbazole		196	---	0.021 ± 0.00035
6	Phenyl	phenyl	198	0.003	0.044 ± 0.0045
19	Phenyl	benzyl	212	---	0.174 ± 0.038
7	Benzyl	benzyl	226	0.0035	0.24 ± 0.0265
18	Butyl	phenyl	178	---	0.635 ± 0.063
3	Allyl	allyl	126	1.1	2.6 ± 0.32
17	Ethyl	benzyl	167	---	2.7 ± 0.084
5	Butyl	butyl	158	0.12	3.5 ± 0.158
22	Dinitrosopiperazine		144	0.57	3.95 ± 0.85
12	Methyl	phenyl	136	0.23	4.0 ± 0.235
16	Ethyl	phenyl	150	---	4.1 ± 0.66
13	Methyl	benzyl	150	0.45	5.55 ± 0.6
21	N-nitrosopiperidine		114	7.7	5.75 ± 0.88
20	N-nitrosopyrrolidine		100	Very sol.	8.0 ± 0.82
2	Ethyl	ethyl	104	10.6	8.0 ± 0.52
8	H	ethyl	74	---	8.7 ± 0.66
14	Ethyl	propyl	116	---	9.05 ± 1.83
15	Ethyl	butyl	130	2.5	9.12 ± 0.915
4	Propyl	propyl	130	0.98	9.5 ± 1.19
9	Methyl	ethyl	88	30	10.1 ± 1.82
1	Methyl	methyl	74	Very sol.	10.2 ± 1.44
10	Methyl	propyl	102	---	10.4 ± 1.67
11	Methyl	butyl	116	---	11.1 ± 1.9

[a] Listed in descending order of activity.

[b] $LD_{100}^{10'}$ = concentration of nitrosamine needed to kill 100% Tetrahymena under light in 10 minutes.

[c] 7,10-DMB(c)A = 7,10-dimethylbenz(c)acridine.

Effects of 4 Doses (4 Days) of Nitrosamines (po) on Body Weight and Pentobarbital Sleeping Time (PST) in Mice

No.	R1 — N-N=O R2 — R1	R2	uM/kg/day (mg/kg/day) 4 days		% change body wt. 5 days	% change PST over control (p)[c]	
	Nitrosamines which prolonged PST						
1	Methyl	methyl	51	(3.75)	12.8	39	(0.05)
9	Methyl	ethyl	86	(7.5)	8.1	69	(0.001)
2	Ethyl	ethyl	144	(15)	14.0	54	(0.001)
10	Methyl	propyl[a]	147	(15)	-3.5	67	(0.001)
11	Methyl	butyl	173	(20)	-22.5	28	(0.06)
14	Ethyl	propyl[a]	215	(25)	0	59	(0.01)
4	Propyl	propyl	308	(40)	12.0	43	(0.01)
13	Methyl	benzyl	333	(50)	-15.0	75	(0.001)
15	Ethyl	butyl[a]	385	(50)	-4.0	98	(0.001)
21	N-nitrosopiperidine		570	(65)	15.2	45	(0.05)
22	N,N-dinitrosopiperazine		694	(100)	14.5	73	(0.001)
20	N-nitrosopyrrolidine		720	(72)	7.5	126	(0.001)
12	Methyl	phenyl	881	(120)	-21.0	39	(0.001)
19	Phenyl	benzyl[a]	2358	(500)	-2.5	29	(0.05)
23	N-nitrosocarbazole[a]		2551	(500)	-0.5	47	(0.02)
5	Butyl	butyl	4430	(700)	-8.0	70	(0.001)
	Nitrosamines which shortened PST (phenobarbital = reference compound)						
24	Phenobarbital Na		15	(3.75)	6.6	-22	(0.02)
3	Allyl	allyl[b]	198	(25)	1.3	-21	(0.05)
17	Ethyl	benzyl[a]	599	(100)	-15.0	-27	(0.001)
16	Ethyl	phenyl[a]	666	(100)	1.0	-23	(0.02)
7	Benzyl	benzyl[b]	885	(200)	5.0	-33	(0.01)
8	H	ethyl[a]	1080	(80)	2.0	-21	(0.05)
18	Butyl	phenyl[a]	1124	(200)	6.5	-15	(0.05)
19	Phenyl	benzyl[a]	1415	(300)	13.3	-22	(0.05)
23	N-nitrosocarbazole[a]		1530	(300)	12.6	-31	(0.02)
6	Phenyl	phenyl[b]	1767	(350)	11.6	-45	(0.01)
5	Butyl	butyl	3797	(600)	4.4	-53	(0.001)

[a]Nitrosamines not yet tested for carcinogenicity.

[b]Known noncarcinogens.

[c]Probability level in Student "t" test. Control animals received olive oil (5 ml/kg/day) po for 4 days. On the fifth day all animals received pentobarbital (100 mg/kg ip).

TABLE 4

Effect of Three Noncarcinogenic Nitrosamines on Pentobarbital Sleeping Time (PST) in Mice[a]

No.	Compound	Dose mg/kg	Duration	% change PST over control group[b]	p[c]
3	Diallylnitrosamine	25	4 days	-21	0.05
		50	4 days	-40	0.001
		100	4 days	-55	0.001
		150	4 days	-45	0.01
		100	2 hrs pretreatment (1 dose)	+71	0.001
		100	24 hrs pretreatment (1 dose)	-57	0.001
6	Diphenylnitrosamine	350	24 hrs pretreatment (1 dose)	-42	0.001
7	Diberzylnitrosamine	200	24 hrs pretreatment (1 dose)	-23	0.07

[a]Dose: 100 mg/kg ip Pentobarbital.
[b]Control group received 5 ml/kg olive oil po.
[c]Probability level in Student "t" test.

TABLE 5

Effect of 2 Hours Pretreatment with a Single Dose Of Nitrosamines (po) on Pentobarbital Sleeping Time (PST) in Mice[a]

No.	uM/kg (mg/kg) po		% change in PST over the control group[d]	(p)[e]
1	676	(50)	66	0.02
2	961	(100)	79	0.001
3[b]	198	(25)	45	0.001
4	1231	(160)	71	0.01
5	3797	(600)	65	0.001
6[b]	1768	(350)	35	0.06
7[b]	885	(200)	59	0.001
8	811	(60)	41	0.001
9	909	(80)	108	0.001
10[c]	980	(100)	95	0.001
11	1034	(120)	63	0.001
12	1684	(229)	98	0.001
13	1333	(200)	92	0.001
14[c]	862	(100)	47	0.01
15[c]	1538	(200)	54	0.02
16[c]	667	(100)	43	0.001
17[c]	559	(100)	60	0.001
18[c]	1124	(200)	65	0.001
19[c]	1415	(300)	59	0.001
20	2000	(200)	63	0.001
21	1754	(200)	9	0.5
22	1389	(200)	43	0.2
23[c]	1531	(300)	45	0.07

[a]Dose: 100 mg/kg ip Pentobarbital.
[b]Known noncarcinogenic nitrosamines.
[c]Nitrosamines not yet tested for carcinogenicity.
[d]Control animals received olive oil 5 mg/kg po before Pentobarbital.
[e]Probability level in Student "t" test.

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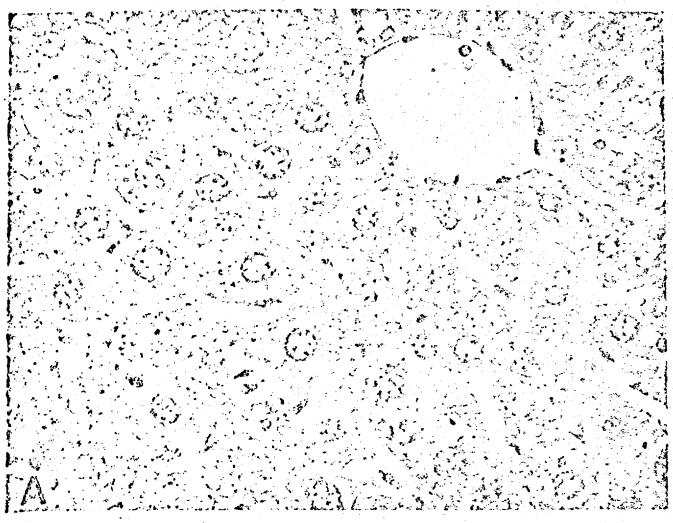
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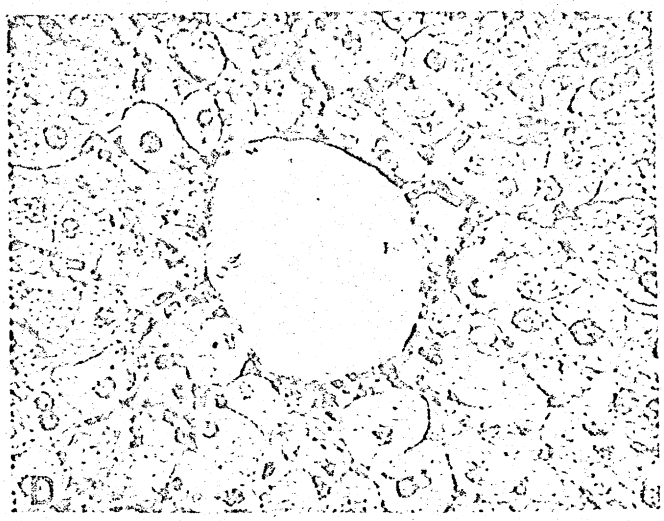
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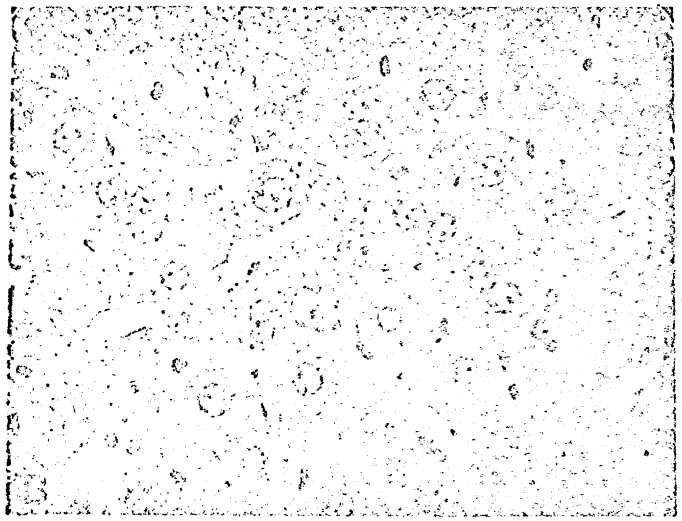

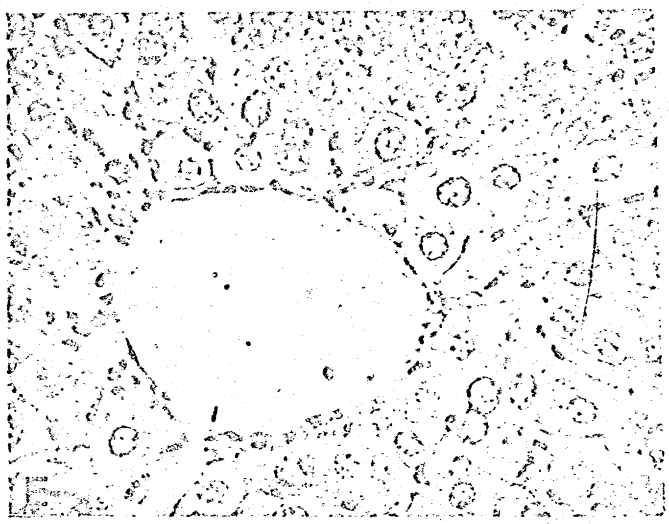
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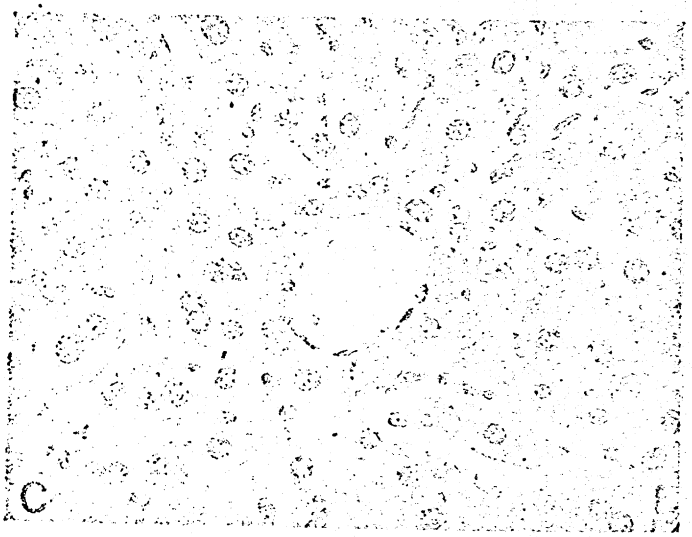
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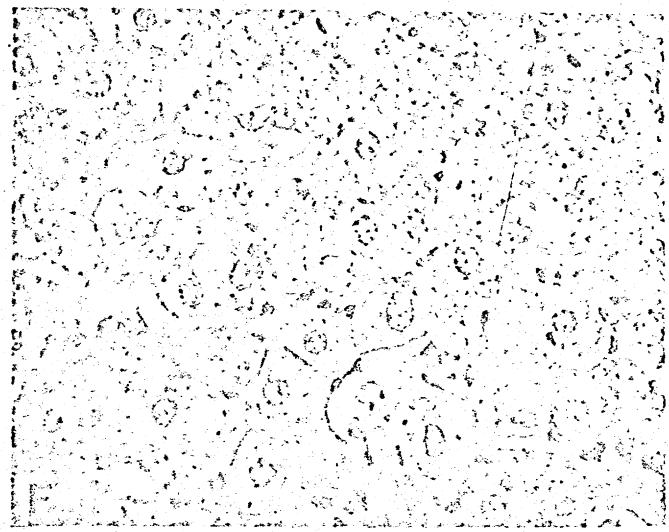
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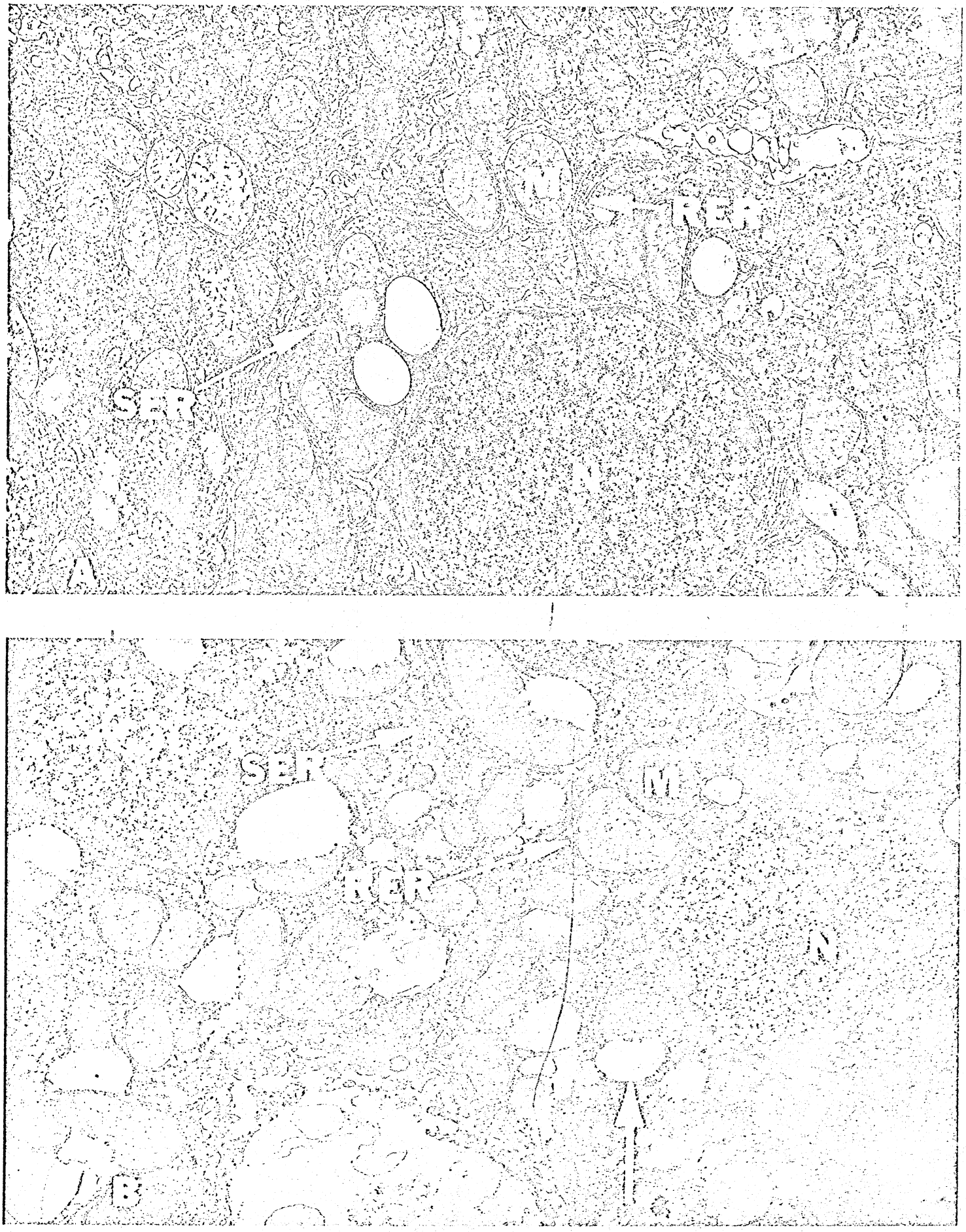
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